JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 2000, p. 444–447
0095-1137/00/$04.00+0

Vol. 38, No. 1

Differentiation of *Mycobacterium tuberculosis* Complex and Nontuberculous Mycobacterial Liquid Cultures by Using Peptide Nucleic Acid-Fluorescence In Situ Hybridization Probes

F. A. DROBNIEWSKI,* P. G. MORE, AND G. S. HARRIS

PHLS Mycobacterium Reference Unit, Department of Microbiology, Dulwich Public Health Laboratory, Guy's King's and St Thomas' Medical School, Dulwich Hospital, London SE22 8QF, United Kingdom

Received 3 August 1999/Returned for modification 10 September 1999/Accepted 28 October 1999

A blinded comparison of peptide nucleic acid-fluorescence in situ hybridization (PNA-FISH) with routine identification methods was performed on 74 consecutively positive mycobacterial liquid cultures. All *Mycobacterium tuberculosis* cultures (48 of 48) and 22 of 27 (81.5%) nontuberculous cultures were correctly identified (including one mixed culture). Five isolates yielded no reaction with either probe and were identified as *Mycobacterium xenopi*, *Mycobacterium fortuitum*, or *Mycobacterium flavescens*.

Eight million new cases of tuberculosis (TB) occur annually in the world, producing almost 3 million deaths, and in 1993, the World Health Organization declared TB to be a global emergency (3). The decline in TB cases in industrialized countries and parts of the developing world ceased or reversed in the mid-1980s. Drug resistance has compromised the successful treatment of TB cases, and multidrug-resistant TB (i.e., having resistance to at least isoniazid and rifampin) has caused high morbidity and mortality, particularly in immunocompromised populations (2, 9). Outbreaks of both drug-sensitive and drug-resistant TB, particularly multidrug-resistant TB, have occurred in both immunocompetent and immunocompromised patients (2, 4, 7, 11). The ability to identify *Mycobacterium tuberculosis* complex (MTC) isolates and differentiate them from nontuberculous mycobacteria (NTM) is therefore of importance for patient management, hospital control of infection, and public health TB control services.

Several in-house and commercial systems for the rapid identification of mycobacterial species isolated from clinical specimens exist, such as the Accuprobe system (GenProbe, San Diego, Calif.), which includes tests for the identification of the MTC, *Mycobacterium avium* complex, *M. avium*, *Mycobacterium intracellulare*, *Mycobacterium kansasii*, and *Mycobacterium gordonae*. The system is highly specific, but a separate test must be performed for each species (8). High-performance liquid chromatography analysis of mycolic acids (6), DNA sequencing of the 16S rRNA gene (10), and 65-kDa *hsp* PCR restriction enzyme assays (12) have been used successfully. Novel commercial systems for the identification of multiple mycobacterial species have been described, including high-density DNA probe arrays on a "microchip" system (5, 13). The latter system has been commercially developed, but the cost of specialized analytical equipment is high.

In most laboratories, smears of putative mycobacterial cultures are prepared to confirm the presence of acid-fast bacilli, detect gross contamination, and, in experienced reference centers, make a tentative separation of MTC and NTM cultures. Unlike solid cultures, only the microscopic appearance of mycobacteria is available for liquid cultures, which is not a reliable distinguishing indicator alone. The culture would normally be identified with a combination of growth and microscopic characteristics, biochemical assays, and high-performance liquid chromatography or DNA hybridization tests (7).

A new commercial culture confirmation system which utilizes the hydrophobic character of peptide nucleic acid (PNA) probes, enabling them to penetrate the mycobacterial cell wall and bind with high specificity to MTC- and NTM-specific rRNA sequences, has been developed. The system detects binding of PNA oligomers (15 oligonucleotides) to MTC-specific and NTM-specific 23S and 16S rRNA sequences, respectively, by a fluorescence in situ hybridization (FISH) system and microscopic techniques familiar to microbiology laboratories (11a).

The aim of the study was to perform a blinded comparison of the DAKO MTC culture confirmation system (Glostrup, Denmark) on liquid mycobacterial cultures from the Organon Teknika MB/BacT 3D nonradiometric culture system (Cambridge, United Kingdom). We prospectively compared the performance of the new PNA-FISH system with conventional methodology used at the United Kingdom Public Health Laboratory Service Mycobacterium Reference Unit. We believe that this is the first study to demonstrate the use of a commercial PNA-FISH system for mycobacterial identification with liquid cultures.

Sequential positive liquid mycobacterial cultures from the continuously monitored and automated Organon Teknika MB/BacT 3D system were used in a prospective blinded analysis. The medium is based on Middlebrook 7H9 broth. Cultures were analyzed by the PNA-FISH assay and then were analyzed conventionally by macroscopic appearance after subculture, microscopic appearance of Ziehl-Neelsen-stained smears, and growth and biochemical characteristics. Identity was confirmed with DNA hybridization systems (Accuprobe; GenProbe) and thin-layer chromatography or PCR-restriction enzyme analysis (1, 8, 12). Drug susceptibility testing was performed by the resistance ratio method on solid Lowenstein-Jensen medium as previously described (1).

The methodology used was as described in the documentation supplied by the manufacturer, DAKO A/S, with modifications. Approximately 1 ml of acid-fast bacillus-positive liquid culture was transferred from the MB/BacT culture vial into a

* Corresponding author. Mailing address: PHLS Mycobacterium Reference Unit, Department of Microbiology, Dulwich Public Health Laboratory, Guy's King's and St. Thomas' Medical School, Dulwich Hospital, East Dulwich Grove, London SE22 8QF, United Kingdom. Phone: 181 693 1312. Fax: 171 346 6477. E-mail: francis.drobniewski@kcl.ac.uk.

TABLE 1. Prospective consecutive samples analyzed by PNA-FISH and conventional methodology

Sample no.	Specimen cultured[a]	Conventional identification	PNA identification	Days to culture	Drug susceptibility[b]
1	Sputum	*M. tuberculosis*	MTC	8	Sensitive*
2	Sputum	*M. malmoense*	NTM	14	
3	Sputum	*M. tuberculosis*	MTC	13	Sensitive
4	Sputum	*M. avium* complex	NTM	6	
5	Sputum	*M. fortuitum*	—[d]	11	
6	Sputum	*M. chelonae*	NTM	7	
7	Sputum	*M. tuberculosis*	MTC	8	Sensitive
8	Sputum	*M. kansasii*	NTM	17	
9	BAL	Scotochromogen	NTM	29	
10	Tissue biopsy	*M. avium* complex	NTM	NA[c]	
11	Sputum	*M. tuberculosis*	MTC	19	Sensitive
12	Pus	*M. avium* complex	NTM	NA	
13	LN	*M. kansasii*	NTM	NA	
14	BAL	*M. tuberculosis*	MTC	11	Sensitive
15	Sputum	*M. tuberculosis*	MTC	14	Sensitive
16	Sputum	*M. tuberculosis*	MTC	10	Isoniazid resistant
17	Sputum	*M. tuberculosis*	MTC	28	Sensitive
18	Sputum	*M. tuberculosis*	MTC	17	Sensitive
19	Sputum	*M. tuberculosis*	MTC	23	Isoniazid resistant
20	Pleural biopsy	*M. tuberculosis*	MTC	26	Sensitive
21	Sputum	*M. tuberculosis*	MTC	9	Sensitive
22	Sputum	*M. xenopi*	—	28	
23	BAL	*M. tuberculosis*	MTC	22	Sensitive
24	Sputum	*M. tuberculosis*	MTC	20	Sensitive
25	Sputum	*M. kansasii*	NTM	9	
26	CSF	*M. tuberculosis*	MTC	27	Sensitive
27	Sputum	*M. malmoense*	NTM	13	
28	Sputum	*M. tuberculosis*	MTC	25	Sensitive
29	Sputum	*M. tuberculosis*	MTC	11	Sensitive
30	Sputum	Scotochromogen	NTM	30	
31	LN	*M. tuberculosis*	MTC	30	Sensitive
32	Sputum	*M. tuberculosis*	MTC	25	Sensitive
33	Sputum	*M. kansasii*	NTM	13	
34	Sputum	*M. tuberculosis*	MTC	6	Sensitive
35	CSF	*M. tuberculosis*	MTC	45	Sensitive
36	BAL	*M. fortuitum*	—	20	
37	Sputum	*M. tuberculosis*	MTC	8	Sensitive
38	Sputum	Scotochromogen	—	18	
39	Sputum	*M. xenopi*	—	25	
40	LN	*M. tuberculosis*	MTC	22	Sensitive
41	Sputum	*M. tuberculosis*	MTC	14	Sensitive
42	Sputum	*M. tuberculosis*	MTC	13	Sensitive
43	BAL	*M. kansasii*	NTM	14	
44	Sputum	*M. kansasii*	NTM	16	
45	Sputum	*M. avium* complex	NTM	11	
46	Pus	*M. tuberculosis*	MTC	27	Sensitive
47	Sputum	Scotochromogen	NTM	29	
48	Sputum	Scotochromogen	NTM	19	
49	Sputum	*M. tuberculosis*	MTC	12	Sensitive
50	BAL	*M. tuberculosis*	MTC	6	Sensitive
51	Sputum	*M. tuberculosis*	MTC	7	Sensitive
52	Sputum	*M. tuberculosis*	MTC	7	Sensitive
53	Sputum	Scotochromogen + *M. tuberculosis*	MTC + NTM		
54	Sputum	*M. tuberculosis*	MTC	6	Sensitive
55	Sputum	*M. kansasii*	NTM	11	
56	BAL	*M. tuberculosis*	MTC	21	Sensitive
57	Sputum	*M. avium* complex	NTM	9	
58	LN	*M. tuberculosis*	MTC	12	Sensitive
59	Pleural fluid	*M. tuberculosis*	MTC	42	Sensitive
60	Sputum	*M. tuberculosis*	MTC	24	Sensitive
61	Psoas abscess	*M. tuberculosis*	MTC	16	Sensitive
62	Sputum	*M. tuberculosis*	MTC	38	Sensitive
63	Sputum	*M. tuberculosis*	MTC	28	Sensitive
64	LN	*M. tuberculosis*	MTC	10	Sensitive
65	BAL	*M. tuberculosis*	MTC	23	Sensitive
66	Pericardial fluid	*M. tuberculosis*	MTC	19	Sensitive
67	Sputum	*M. tuberculosis*	MTC	9	Sensitive

Continued on following page

TABLE 1—*Continued*

Sample no.	Specimen cultured[a]	Conventional identification	PNA identification	Days to culture	Drug susceptibility[b]
68	BAL	*M. chelonae*	NTM	5	
69	Pus	*M. tuberculosis*	MTC	17	Sensitive
70	Sputum	*M. tuberculosis*	MTC	13	Sensitive
71	Sputum	*M. tuberculosis*	MTC	8	Sensitive
72	BAL	*M. avium* complex	NTM	9	
73	Sputum	*M. tuberculosis*	MTC	12	Sensitive
74	BAL	*M. tuberculosis*	MTC	8	Sensitive

[a] LN, lymph node; BAL, broncho-alveolar lavage; CSF, cerebrospinal fluid.
[b] Drug susceptibility testing on *M. tuberculosis* cultures by the standardized resistance ratio method (1) for isoniazid, rifampin, ethambutol, pyrazinamide, and streptomycin. *, sensitivity to five drugs tested.
[c] NA, data not available; cultures were submitted from a second laboratory.
[d] —, not identified by DNA system.

clean screw-top Microfuge tube and centrifuged at 13,000 × *g* for 5 min (model no. 5417C; Eppendorf, Hamburg, Germany). The supernatant was carefully removed, 100 to 200 μl of phosphate-buffered saline was added to each tube, depending on the size of deposit visible, and the contents were mixed. From this, 25 μl was added to each of two wells on the supplied two-well slide. The slide was allowed to air dry (approximately 30 min) before fixing the smears by passing the slide through a Bunsen burner flame three or four times and then immersing it in 80% (vol/vol) ethanol for 15 min. The slide was then air dried for a further 10 min. One drop of the fluoroscein isothiocyanate (FITC)-labelled MTC PNA conjugate was added to one well, and one drop of FITC-labelled NTM PNA conjugate was added to the other well. A coverslip (22 by 22 by 0.15 mm) was added to each well, and the slide was incubated in a humidity chamber for 90 min at 55°C. The wash solution was prepared and preheated to 55°C. The coverslips were removed, and the slides were immersed in wash solution for 30 min at 55°C. They were then dipped in distilled water for 30 s and air dried for 10 min. One drop of mounting fluid was added to each well, and the wells were covered with a double coverslip (24 by 60 by 0.15 mm) and incubated in a humidity chamber for 30 min at 55°C. The smears were examined with a fluorescent microscope equipped with a FITC-Texas Red dual-band filter set and a 100× oil objective (Hund) (5 min per slide) (total magnification, ×1,000). Mycobacteria were detected on the basis of green fluorescence against a red-brown background and characteristic morphology. MTC appears as a 1- to 3-μm slender, rod-shaped bacillus which may show cording. NTM species vary in morphology. The intensity of fluorescence may vary, depending on growth medium and species.

The results are given in Table 1. A total of 74 cultures were prospectively analyzed by the usual reference laboratory procedures; 47 cultures were shown to be MTB alone, one was a mixture of MTC and NTM, and 26 were NTM alone. The mean time taken to culture *M. tuberculosis* from all specimens and from sputum isolates was 17.6 days (range, 6 to 45 days) and 15.2 days (range, 6 to 38 days), respectively. Nontuberculous isolates were cultured for a mean of 16 days (range, 5 to 30 days). The PNA-FISH system correctly identified all MTB cultures (48 of 48), and 22 of 27 (81.5%) NTM cultures were correctly identified as NTM. Two *M. tuberculosis* strains which subsequently showed isoniazid resistance were also correctly identified by the PNA system, which is of importance since resistance is associated with changes in mycolic acid synthesis and bacterial surface properties. The five NTM cultures which gave no reaction with either of the two probes were identified as *Mycobacterium xenopi*, *Mycobacterium fortuitum* (2), and *Mycobacterium flavescens*. Overall, no result was obtained in 5 of 74 (6.8%) total cultures flagged positive for mycobacteria.

In general, the new culture confirmation system performed well, identifying all *M. tuberculosis* cultures analyzed. A weak signal was seen in 7 of the 48 MTC-containing cultures (14.6%), although in half the cultures, few mycobacteria were seen by direct microscopy with Ziehl-Neesen staining. Five NTM cultures were not identified by PNA-FISH. Earlier data obtained with pure cultures had suggested that the NTM probe was not complementary to rRNA from *Mycobacterium marinum*, *M. xenopi*, *M. flavescens*, and *M. fortuitum*, so these species would not be recognized by the assay (11a). The NTM probe is complementary (and so cross-reacts) to rRNA from species of *Actinomyces* and *Rickettsia* (11a). Neither probe cross-reacts with rRNA from *Corynebacterium* or *Neisseria* species or from *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Propionibacterium acnes*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Moraxella catarrhalis*, *Escherichia coli*, and *Nocardia asteroides* (11a).

The new system is principally targeted at the niche occupied by the Accuprobe system, which is able to identify cultures of the MTC, the *M. avium-M. intracellulare* complex, *M. avium*, *M. kansasii*, and *M. gordonae*. Neither the new assay nor the Accuprobe system is able to differentiate between members of the MTC or identify all NTM isolates. The fluorescent assay is able to identify a broader range of NTM species than the Accuprobe but cannot distinguish between species. It does permit direct visualization of mycobacteria which, in the hands of experienced reference staff, can support the provisional identification of MTC by macroscopic and microscopic appearances even when low fluorescence due to a small organism load is a problem. Routine clinical laboratories examining few mycobacterial cultures may have difficulty with tests yielding low fluorescence, which was a problem with some cultures, particularly NTM. The test, however, does allow for a second opinion in tests exhibiting borderline results, in that the slides can be reviewed. The identification of mixed mycobacterial cultures is also possible, as occurred in this study. It is less likely that smaller clinical laboratories seeing few mycobacterial cultures would be as successful, as low fluorescence was the most consistent problem with the FISH, particularly when examining NTM. The systems use comparable levels of general laboratory equipment; a luminometer is required for the Accuprobe system, and an appropriately modified FITC-Texas Red filter set needs to be fitted to the microscope to obtain reproducible results with the FISH assay. These additional costs would probably prohibit small laboratories and those in the developing parts of the world from utilizing the FISH assay.

The overall times needed to perform the assays are comparable.

In conclusion, the new FISH assay offers a useful alternative to current methods for identifying MTC cultures in reference laboratories.

DAKO TB Probe Culture Confirmation Kits were supplied by DAKO A/S. We acknowledge the kind support and helpful discussions of Henrik Stender and Ole Rasmussen.

The study was funded by DAKO A/S and the UK Public Health Laboratory Service.

REFERENCES

1. **Collins, C. H., J. M. Grange, and M. D. Yates.** 1997. Tuberculosis bacteriology, organisation and practice, 2nd ed. Butterworth-Heineman, Oxford, United Kingdom.
2. **Drobniewski, F. A.** 1997. Is death inevitable with multi-resistant TB plus HIV infection? Lancet **349**:71–72.
3. **Drobniewski, F. A., A. Pablos-Mendes, and M. R. Raviglione.** 1997. Epidemiology of tuberculosis in the world. Semin. Respir. Crit. Care Med. **18**:419–429.
4. **Fischl, M. A., R. B. Uttamchandani, G. L. Daikos, R. Poblete, J. Moreno, R. Reyes, A. M. Boota, L. M. Thompson, T. J. Cleary, and S. Lai.** 1992. An outbreak of TB caused by multi-drug resistant tubercle bacilli among patients with HIV infection. Ann. Intern. Med. **117**:177–183.
5. **Gingeras, T. R., G. Ghandour, E. Wang, A. Berno, P. M. Small, F. Drobniewski, D. Alland, E. Desmond, M. Holodniy, and J. Drenkow.** 1998. Simultaneous genotyping and species identification using hybridization pattern recognition analysis of generic Mycobacterium DNA arrays. Genome Res. **8**:435–448.
6. **Glickman, S. E., J. O. Kilburn, W. R. Butler, and L. S. Ramos.** 1994. Rapid identification of mycolic acid patterns of mycobacteria by high-performance liquid chromatography using pattern recognition software and a *Mycobacterium* library. J. Clin. Microbiol. **32**:740–745.
7. **Goble, M., M. D. Iseman, L. A. Madsen, D. Waite, L. Ackerson, and C. R. Horsburgh.** 1993. Treatment of 171 patients with pulmonary tuberculosis resistant to isoniazid and rifampin. N. Engl. J. Med. **328**:527–532.
8. **Heifets, L. B., and R. B. Good.** 1994. Current laboratory methods for the diagnosis of tuberculosis, p. 85–110. *In* B. R. Bloom (ed.), Tuberculosis protection, pathogenesis, protection, and control. American Society for Microbiology, Washington, D.C.
9. **Iseman, M. D.** 1993. Treatment of multidrug-resistant tuberculosis. N. Engl. J. Med. **329**:784–791.
10. **Kirschner, P., and E. C. Böttger.** 1998. Species identification of mycobacteria using rDNA sequencing, p. 349–361. *In* T. Parish and N. G. Stoker (ed.), Mycobacteria protocols. Humana Press, Totowa, N.J.
11. **Small, P. M., R. W. Shafer, P. C. Hopewell, S. P. Singh, M. J. Murphy, E. Desmond, M. F. Sierra, and G. K. Schoolnik.** 1993. Exogenous reinfection with multi-drug resistant Mycobacterium tuberculosis in patients with advanced HIV infection. N. Engl. J. Med. **328**:1137–1144.

11a. **Stender, H., K. Lund, K. H. Petersen, O. F. Rasmussen, P. Hongmanee, H. Miörner, and S. E. Godtfredsen.** 1999. Fluorescence in situ hybridization assay, using peptide nucleic acid probes for differentiation between tuberculous and nontuberculous mycobacterium species in smears of mycobacterium cultures. J. Clin. Microbiol. **37**:2760–2765.

12. **Telenti, A., F. Marchesi, M. Balz, F. Bally, E. C. Böttger, and T. Bodmer.** 1993. Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis. J. Clin. Microbiol. **31**:175–178.
13. **Troesch, A., C. Nguyen, C. G. Miyada, S. Desvarenne, T. R. Gingeras, P. M. Kaplan, P. Cros, and C. Mabilat.** 1999. *Mycobacterium* species identification and rifampin resistance testing with high-density DNA probe arrays. J. Clin. Microbiol. **37**:49–55.